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ISOLATION AND IDENTIFICATION OF SEAWATER BACTERIA WITH SUPEROXIDE DISMUTASE ACTIVITY FROM THE PASIR PUTIH BEACH

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ABSTRACT
Reactive Oxygen Species (ROS) are free radicals responsible for degenerative diseases and cellular aging, which can be neutralized by enzymatic antioxidants such as Superoxide Dismutase (SOD). Marine bacteria residing in photic coastal zones represent a promising source of stable SOD due to continuous solar and oxidative stress. This study aimed to isolate and identify seawater bacteria from Pasir Putih Beach, Situbondo, East Java, and evaluate their cellular SOD activity, protein concentration, and subunit molecular weight. Seawater samples collected via purposive sampling were isolated on Nutrient Agar (NA) media and characterized morphologically, microspically via Gram staining, and biochemically. Crude SOD enzymes were extracted by sonication, partially purified using 60% ammonium sulfate precipitation, characterized by SDS-PAGE, and quantified for protein content using the Lowry assay. SOD antioxidant activity was evaluated via WST-1 assay for percent inhibition. Eight bacterial isolates (BAL 01–BAL 08) were successfully isolated. Isolate BAL 06 demonstrated the highest SOD enzyme activity, exhibiting an inhibition value of 86.07%, a total protein content of 11.88 mg/mL, and an estimated molecular weight of 30 kDa on SDS-PAGE. This research provides significant scientific contributions by discovering potential marine bacterial strains as accessible, high-yield biological sources of natural enzymatic antioxidants for biotechnological and pharmaceutical applications.

Keywords: antioxidants; free radicals; marine bacteria; superoxide dismutase; WST-1 assay

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INTRODUCTION

Indonesia is a country with high sun exposure which is one of the factors causing an increase in free radicals in the body. Free radicals are unstable molecules with high reactivity because they have unpaired electrons and can react with other molecules and result in harmful chain reactions (Wimpie P, 2017). ROS can be neutralized with endogenous antioxidants, both non-enzymatic and enzymatic, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR). The therapeutic potential of SOD enzymes can function as anti-inflammatory agents and can prevent precancerous cell changes. The natural SOD levels in the body decrease as the body ages and because of this a person becomes more susceptible to diseases related to oxidative stress. SOD is used in cosmetics and care products as an anti-aging and antioxidant ingredient due to its ability to reduce damage.

Pure SOD enzymes have a fairly expensive price so research was carried out to look for SOD-producing bacteria, then stated that the bacteria *Deinococcus radiodurans* is one of the bacteria that can produce the enzyme SOD. When the enzymes of this bacterium were tested on the biosensor, it turned out to be a SOD-based antioxidant biosensor from a protein extract *Deinococcus radiodurans* The results of the study stated that marine bacteria *Geobacillus sp.* isolated from the sea shows high thermostability. In the study it was found that recombinant SODs from *Geobacillus sp* produces 437 amino acid residues. Recombinant enzymes have high thermostability at 50oC which indicates that SOD is resistant to hot temperatures (Wimpie P, 2017) SOD can be found in bacteria, *Archeae* and *Eucarya* (Sheng *et al.*, 2014)produces a high affinity of substrate enzymes

rather than pure SOD (Iswantini *et al.*, 2019).

Research on 3 types of bacteria *Cyanobacteria*, *Synechocystis sp*, *G. violaceus* and *Geitlerinema sp* shows FeSOD activity on *Synechocystis sp*, while in *G. violaceus* and *Geitlerinema sp* FeSOD 1 and FeSOD2 activity was found (Kula, 2022). On the isolation of soil bacteria *E.coli* It was found to have a recombinant GsSOD with a protein molecular weight of ~30 kDa that showed the highest activity at 10oC and pH 7.8. GsSOD was able to tolerate temperatures of 100oC and 130oC and was stable at various concentrations of chemicals such as potassium cyanide, H2O2 and chloroform (Guleria *et al.*, 2021).

The results of the study stated that marine bacteria *Geobacillus sp.* isolated from the sea shows high thermostability. In the study it was found that recombinant SODs from *Geobacillus sp* produces 437 amino acid residues. Recombinant enzymes have high thermostability at 50oC which indicates that SOD is resistant to hot temperatures (Zhu *et al.*, 2014). SOD can be found in bacteria, *Archeae* and *Eucarya* (Sheng *et al.*, 2014). FeSOD can be found in chloroplasts and peroxisomes, MnSOD is located in mitochondria and peroxisomes, while Cu/ZnSOD is detected in chloroplasts, mitochondria, nuclei and extracellular matrices (Sheng *et al.*, 2014). Based on this description, the researcher is interested in conducting research on the activity of SOD enzymes in marine bacteria as an antidote to free radicals, this is the background for a study entitled "Isolation and Identification of Seawater Bacteria with *Superoxide Dismutase Activity* from the Pasir Putih Beach, East Java"

METHOD

Design, place and time

The research was conducted at the microbiology laboratory of Setia Budi University of Surakarta and the Biotechnology Laboratory of Gajah Mada University Yogyakarta from July to December 2023.

Types and Methods of Data Collection

In this study, the research population carried out by the *purposive sampling*. Seawater samples were taken at the Pasir Putih beach of Situbondo. Seawater sampling was carried out during the day when the weather is sunny and at a temperature of 37ºC with a depth of 10 cm and then put into a sterile glass bottle (100 ml) and put in a *Coolbox* to avoid contamination. After that, the samples were taken to the Microbiology Laboratory of Setia Budi University Surakarta to be isolated for bacterial isolation.

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Data Processing and Analysis

Bacterial Isolation

Bacterial isolation by carrying out a dilution process first by means of 1 ml of seawater sample suspended using 9 mL NaCl and then vortex until homogeneous. The result of the suspension is taken 1 ml and then suspended back into 9 mL NaCl and referred to as dilution factor 10^{-1}, the same step is repeated up to a dilution factor of 10^{-2} and 10^{-3} (Sapphire) *et al.*, 2019). At dilution 10^{-3} isolation was carried out to obtain bacterial isolates by scratching with an OSE needle aseptically on the NA media that had been prepared. Then it was incubated for 24 hours at a temperature of 37oC until the bacterial colony grew.

Identification of Bacteria by Gram Staining

Gram staining on bacteria is done by sterilizing the glass object by putting it in a 70% alcohol solution then wiping it with a cotton swab until it emits a squeaking sound. Circle the bottom of the glass object with a marker as an area for applying the bacteria sample. The ose needle is fixed by burning it with a spirtus burner until the ose is incandescent. The ose is cooled by being brought closer to the spirtus burner. The preparation of bacterial smear is made by mixing one bacterial culture ose with sterile aqua dest, then making the smear as thin as possible, dried and fixed on a

spirit lamp. Then place the glass object on a color bath, flood the smear with violet fiber carbol dye for 1 minute, remove the excess dye, then rinse with distilled water. Flood the spread with lugol for 2 minutes, remove the excess lugol and rinse with distilled water. Wash the spread with 95% alcohol drop by drop for 30 seconds or until the dye dissolves, then rinse with distilled water. Flood the spread with fuxin water dye for 30 seconds, remove the excess color, rinse with distilled water and dry with filter paper. Drip a small amount of emerment oil on the glass object, then check under a microscope with 100x magnification (Krishna, 2013).

Identification of Bacteria by Biochemical Test

Biochemical activity is a variety of chemical reactions that take place in the body of living things to sustain life. Biochemical tests include catalase tests, coagulase tests, driver's license tests, SAC tests, and driver's license tests.

Extract Enzyme *Superoxida Dismutase* (SOD)

Bacteria as much as 2 ose from oblique agar were put into a 10 ml BHI medium and then incubated at a temperature of 37oC for 18-24 hours, taken as much as 2 ml and put into an Erlenmeyer containing 400 mL of BHI liquid media followed by the incubation stage at 37oC for 18-24 hours. Then it was centrifuged to separate the pellets and the supernatant. The centrifugation process was repeated until the solution became clear. The pellets obtained were weighed and added with a PBS ratio of 1:2 and re-centrifugated. The pellets were continued at the breaking stage mechanically using sonication at a cold temperature with an amplitude of 50 for 15 minutes with intervals of every 1 minute at a temperature of 4oC. After the sonication stage, it is continued in the centrifugation stage at 5000 rpm for 60 minutes to obtain supernatant which is a coarse extract of the SOD enzyme. The results of the enzyme extraction are stored at a temperature of 4oC for 24 hours in a refrigerator.

Molecular Mass Determination of SDS PAGE

The coarse extract of the enzyme obtained was then characterized to determine the molecular weight using the SDS PAGE method. The molecular weight of the sample protein was measured by SDS PAGE electrophoresis. The sample was characterized using SDS-PAGE first mixed in a 1:1 ratio. The mixture between dialysate and the sample buffer was boiled over a water bath for 5 minutes to reduce the protein to denature, then the denatured protein was cooled at room temperature for 15 minutes before being put into a gel well.

Protein Levels Measurement with the Lowry Method

Manufacture of Lowry reagent. There are 4 reagents, namely reagent A, reagent B, reagent C, and µL B reagent. Reagent A is made by dissolving 2g Na2CO3 in 100 ml NaOH 0.1N. In reagent B, it is made by mixing 5 ml of 1% CuSO4.5H2O with 5 ml of 2% NaK tartrate solution. In reagent C, it is made by mixing 2 ml of Lowry B reagent into 100 ml of Lowry A. In reagent D, it is made by diluting *folin phenol ciocalteau* with aquadest with a ratio of 1:1 v/v.

Manufacture of protein standard solution. 50 mg of BSA is dissolved into aquadest ad 50 ml so that a standard parent solution with a concentration of 1000 ppm is obtained. The standard curve is made by diluting the concentration parent solution of 1000 ppm until 6 concentration series are obtained, namely 20, 40, 60, 80, 100, and 120 ppm.

Determination of maximum wavelength. A standard solution of proteins is placed into a cuvette and then measured at its maximum wavelength in the wavelength range of 400-800 nm. Aqua distillata is used as a blank, and the highest adsorbent value is used as the maximum wavelength.

Determination of *operating time*. The standard solution of protein is read absorbance at the maximum wavelength starting from minute 0 to minute 45 until a stable absorbance value is

obtained

Creation of standard curves
Each concentration series is taken 1 ml and then put into the test tube, then 5 ml of Lowry C reagent is added and left for the *operating time*. Lowry D reagent is then added to each sample by 0.5 ml and shaken so that the final volume of the solution is 6.5 ml and left for 10 minutes. The absorbance is read at the maximum wavelength obtained. The concentration and absorbance values are plotted until the linear regression equation y = a+bx is obtained.

Measurement of protein levels of bacterial SOD extract
A sample of 1 ml was added to 5 ml of Lowry C reagent and Left to fit *operating time.* 0.5 ml of Lowry D reagent added. After that, it is shuffled and left for 10 minutes or until a blue color is formed. The absorbance is read at the maximum wavelength obtained. The absorbance value is plotted into a linear regression equation and can be used for the calculation of sample protein levels.

SOD Enzyme Activity Testing with WST 1 Method
Testing of the activity of SOD enzymes was carried out to determine its ability to catalyze dismutase from superoxide anions into hydrogen peroxide molecules, the use of *SOD assay kits* was able to produce formazan colors. Formazan is water-soluble based on the reduction of superoxide anions.

Preparation of WST *working solution*. A total of 1 ml of WST solution is diluted with 19 ml of *dapar assay solution*. This solution is stable at a temperature of 4oC for 2 months.

Preparation *of enzyme working solution*. Centrifugation of enzyme solution for 5 seconds. The solution is mixed evenly using a pipette. An enzyme solution of 15 µl is diluted with 2.5 dapar solutions. The result of dilution of the enzyme solution is stable for up to 3 weeks at a temperature of 4°C.

Preparation of the test sample. A total of 20 µl of sample solution was added to each sample and blank well 2, while 20 µl of ddH20 (double distilled water) was added to each of blank 1 and blank well 3. 200 µl of WST was added to each well and then mixed, in each of blank wells 2 and 3 20 µl of dilution *buffer* was added. Then 20 µl of enzyme working solution was added to each sample and blank well 1, then stirred until homogeneous, the plates were then incubated at 37oC for 20 minutes. Absorbance was read at a wavelength of 450 nm using a microplate. The absorbance results were used to determine the percent inhibition using the formula:

$$\% \text{ Inhibisi} = \frac{(A \text{ blank1} - A \text{ blank3}) - (A \text{ sample} - A \text{ blank2}) \times 100\%}{A \text{ blanco1} - A \text{ blanko3}}$$

With a caption:
A blanko 1 : Absorbansi blanko 1
A blanko 2 : Absorbansi blanko 2
A blanko 3 : Absorbansi blanko 3
A sample: Absorbance of sample

RESULT
Seawater Sampling
The seawater sample used in the study was taken in March 2023 at a temperature of 37oC. As much as 100 mL and put in a glass bottle that has been sterilized first, sampling is carried out at one point to get accurate and valid results with a depth of 10 cm which aims to prevent dirt on the seabed from being carried into the bottle.

Seawater Bacteria Isolation and Identification

The results of the isolation of seawater bacteria showed that there were eight different isolates including size, shape, colony edges, elevation and color.

Identification of Bacteria by Gram Staining

Bacterial staining in this study needs to be done evenly so that bacteria do not accumulate and facilitate microscopic observation. The staining results of Gram isolates BAL 01, BAL 02, BAL 03, BAL 05, BAL 06, BAL 7 and BAL 08 are classified as gram positive with a round shape (*Coccus*), while for BAL 03, BAL 06, BAL 07 and BAL 08 are rod-shaped (*Bacil*).

Biochemical Activity Testing

Identification was continued by conducting biochemical tests in the form of coagulage, catalase, SCA, LIA, and KIA and SIM tests. In this study, only BAL 05 was positive for coagulase enzymes. Catalalase tests on the isolates of BAL 01, BAL 02, BAL 03, BAL 04, BAL 07, and BAL 08 showed positive results containing catalase which was characterized by the formation of gases. The next stage of the biochemical test is the *simmon's citrate* which shows BAL 01, BAL 02, BAL 03, BAL 05, BAL 6 and BAL 7 were positive, while BAL 04 and BAL 08 were negative. The biochemical test of LIA showed that BAL 01 was negative for LIA, while the seven isolates showed positive results marked by the color of the media changing to purple. The results of the KIA media test were positive if there were deposits at the base of the media and eight isolates showed positive results (Sineb & Gergonius, 2014). In this study, sulfur was positive only in BAL 01 while in BAL 02 to BAL 08 it was negative, and for motility all bacterial isolates showed positive results. The Indole test in this study showed all indole negative bacteria,

Extractive Enzyme SOD

The extraction of an enzyme has the purpose of removing enzymes in bacteria and separating enzymes from impurities. The results of SOD enzyme extraction using BHI 400 mL media were obtained as in the following table 1:

Table 1.
Results of enzyme extraction of seawater bacteria

Isolation	Sample volume (mL)	Sediment (g)
BAL 01	400	8,100
BAL 02	400	7,080
BAL 03	400	7,028
BAL 04	400	8,765
BAL 05	400	9,142
BALL 06	400	8,465
BALL 07	400	9,098
BALL 08	400	6,209

SDS PAGE Results

The characterization of SOD includes the determination of the total molecular weight of its subunits, amino acid composition and metal cofactors. The total molecular weight and its subunits are carried out using SDS-PAGE with a concentration of 10% separating gel and 4% acrylamide gel stacking (Tri Panji, *et al.* 2022). The results of the study show that the resulting SOD extract has a molecular weight of 30 kDa as shown in the following figure:

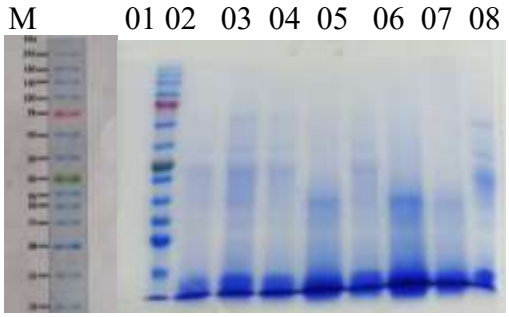


Figure 1. (A) Marker (B) SDS PAGE results after purification

Precipitation with Ammonium Sulfate

The extracted SOD enzyme is then purified with ammonium sulfate, enzyme purification is carried out with the aim of obtaining the specific enzyme desired and removing some impurities such as carbohydrates (F.Fitria, 2017). The results of ammonium sulfate precipitation are shown in Table 2.

Table 2.
Results of enzyme precipitation with ammonium sulfate 60%

Isolate	Sediment (g)
BALL 01	6,460
BALL 02	5,403
BALL 03	5,531
BALL 04	6,698
BALL 05	7,605
BALL 06	7,843
BALL 07	7,931
BALL 08	4,953

This study uses a precipitation method with 60% ammonium sulfate salt. Research Trias Ayu, 2022 mentions that Protein levels and enzyme activity increased from deposition using 0% ammonium sulfate to 60%, then decreased activity in deposition using ammonium sulfate 80%.

Protein Levels Measurement with the Lowry Method

Determination of the protein content of the crude extract of the SOD enzyme of seawater bacteria was carried out using the Lowry method. The measurement of total protein content was carried out to see the effect of the extract on enzyme production or on enzyme activity. Enzymes in the body include the enzyme CAT (*Catalase*). GSH-Px (*glutation peroksidase*) (Nurfitriyawatie, 2023). The results of the protein content test in this study can be seen in table 09 as follows:

Table 3.
Protein Test Results

Isolate	Protein (mg/mL)
BALL 01	4,28
BALL 02	2,34
BALL 03	3,30
BALL 04	8,29
BALL 05	5,48
BALL 06	11,88
BALL 07	9,21
BALL 08	1,72

The results of this study show that the bacteria with the highest protein content is BAL 06 of 11.88 mg/mL. According to Fardiaz (1992), the better the nutrients in the substrate where they grow, the faster the cell growth which will increase the protein content of the cell. In addition, the protein content of cells is influenced by the reproduction time. According to Kuswardani and Wijajaseputra (1998), the reproduction time that is too short will produce low amounts of protein because the bioconversion of the medium components is not optimal. Meanwhile, too long the reproduction time will cause a decrease in accumulated protein.

SOD Enzyme Activity with WST-1

The SOD enzyme activity test was carried out using the WST-1 method, this method was chosen because the solubility of salts in water is better than the NBT method, this method is based on the cleavage of tetrazolium salt WST 1 into formazan by *dehydrogenase* cellular mitochondria. The greater the number of living cells, the higher their activity and the greater the amount of formazan dyes formed (Magda Jeane, 2018). The results of SOD activity testing are shown in table 3, as follow:

Table 3.
WST Test Results 1

Isolate	Inhibisi (%)
BALL 01	35,25
BALL 02	45,90
BALL 03	61,48
BALL 04	85,59
BALL 05	40,00
BALL 06	86,57
BAL 07	85,07
BAL 08	46,72

The SOD activity test was divided into 4 parts, namely samples (samples, enzymes, and WST), blank 1 (enzymes, WST and H20), blank 2 (buffer, sample and WST), and balnk 3 (WST, sample, buffer, H20). SOD activity has high activity criteria if it has an inhibition percentage of >50% and low activity if the inhibition percentage is <50% (Nurfitriyawatie, 2023). The % inhibition value indicates total SOD activity is based on the O2-reduced nitroblue tetrazolium (NBT) inhibition. One unit of SOD is defined as the amount of enzymes that cause 50% inhibition at the NBT reduction rate.

DISCUSSION

Seawater Bacteria Isolation and Identification

Bacterial isolation aims to separate one type of microbe from another from various kinds of microbial mixtures with the aim of obtaining pure cultures (Safitri *et al.* , 2019) . In this study, a multi-stage dilution was carried out which aims to dilute the microorganisms in the sample to be scratched on the agar medium. The results of isolation were obtained from several growing colonies, the colonies were observed macroscopically based on their morphology so that eight single colonies were obtained

Identification of Bacteria by Gram Staining

The principle of Gram staining is the difference in the components of the bacterial cell wall, the size of the peptidoglycan content and the presence of the outer membrane which affects the ability of the cell wall to bind to the basic color of CV (*Crystal Violet*), after washing with alcohol (Amen *et al.*, 2023). Gram-positive bacterial cells that do not contain an outer membrane in the form of fat, their dominant walls contain peptidoglycan will bind to CV firmly after being fortified with Iodine (Amen *et al.*, 2023). Meanwhile, CV that is bound to the outer membrane of Gram-negative cells will be washed (dissolved) by alcohol. After the base color is washed in Gram-negative bacteria, the comparator color given (safranin) will be bound by the cell, so that the cell is colored pink. Meanwhile, Gram-positive bacteria that predominantly contain peptidoglycan will still retain the color of CV (CV-I complex) which binds to peptidoglycan because alcohol wrinkles the cell so that the CV-I complex enters the cell wall more optimally (Zulfarina *et al.*, 2022).

BAL 04 isolate is classified as Gram-negative and is rod-shaped (*bacill).* It is characterized by red cells because the ability of bacteria to bind to saffron is very strong (Cappucino *et al*., 1998). The cell wall components of gram-negative bacteria are mostly composed of lipid layers, so that at the time of staining it is less able to retain the main dye, especially when washed with alcohol (lipids

are damaged when washed with alcohol) as a result of this group of bacteria looks red in the results of Gram staining.

Biochemical Activity Testing

The coagulase test is based on the principle of detecting the presence of the coagulase enzyme in the bacteria tested. Coagulase is an enzymatic protein that functions to convert fibrinogen into fibrin which causes freezing or clumping (Jamilatun, 2019). Serum coagulase reaction factors react with coagulates to produce esterase and clotting activity in the same way, as does the activation of prothrombin into thrombin. In the catalase test, it can be stated positive if the isolate dripped with H2O2 causes bubbles (Pulungan & Tumangger, 2018). Bacterial isolates that do not produce bubbles are declared as catalase negative. This proves that the given H2O2 cannot be broken down by bacteria so that it does not produce oxygen. Cathalase-negative bacteria do not have the enzyme catalase that breaks down H2O2 (Cappucino, J. G., 2001). The mechanism of the catalase enzyme to break down H2O2 is that when respirating, bacteria produce various components, one of which is H2O2. Bacteria that have the ability to break down H2O2 with the catalase enzyme immediately form a defense system from the toxic H2O2 produced (Zulfarina *et al.*, 2022). Media SIM has a BTB indicator (*Brom Tymol Blue*). If bacteria use citrate as a source of carbon, the pH of the medium will change to alkaline and is shown by the change in wana from green to blue. The ability of bacteria to hydrolyze citrate is due to the fact that bacteria are able to produce the enzyme citrate permease which is a specific enzyme that carries citrate into the cell (Rahmiati, 2019).

On a positive KIA test, it shows Microorganisms that have the ability to decarboxylate lysine in the medium will remain purple in color during alkaline conditions, while microorganisms that deaminize lysine will produce yellow color during acidic conditions (Toelle, *et al.*2013). The KIA test aims to test the fermentation of carbohydrates, namely lactose and glucose, as well as sulfide. The ability of bacteria to convert dextrose and lactose as well as the ability to produce hydrogen sulfide is the basis for determining the type of bacteria from their growth in the medium, the presence of gas produced from the glucose fermentation process is indicated by the presence of air bubbles in the media. The SIM media used is semi-solid or semi-agar, and in the SIM test there are three stages of the test, namely, sulfur, indole and motil. Sulfur is hydrogen sulfide (H2S). If there is a black deposit at the base, it means sulfur (+) is positive. In indole, if it is positive, there is a layer of red rings on the surface. While motile is marked if there are traces of bacterial movement such as the presence of white fibers in the inoculation marks of the sim media (Sineb & Gergonius, 2014). The principle is that an indole test is carried out to determine the ability of bacteria to break down the amino acid tryptophan to form the compound Indol. Tryptophan is hydrolyzed by tryptophane and produces three products, one of which is indole. The production of this indole is detected by the Kovac reagent and reacts to produce a red compound (Rifai, 2021). Indole can be identified by the complex reaction of a red dye with one of several reagents, e.g. *Kovac's Reagent* which consists of *amyl alcohol*, *para-dimethylaminobenzaldehyde* and concentrated hydrochloric acid.

Extractive Enzyme SOD

This study produced different supernatant and sediment weights of each bacterium. The weight of supernatant and pellets has different weights due to different metabolic processes of each bacterium (Zhu *et al.*, 2014). The centrifugation process to obtain cell-free supernatant so that the cell will settle due to the force of gravity and obtain different sample weights (Zulfarina *et al.*, 2022). The centrifugation process has the purpose of separating the supernatant which is suspected to be a coarse extract of the enzyme from the remains of the fiber in green spinach. The centrifugation process is carried out at low temperatures so that the enzyme activity can be maintained as much as possible (Masruroh *et al*, 2018). The filtrate from the centrifugation process will produce two different parts, namely supernatant and sediment (pellet). The extracted part is the supernatant that will be used for the partial purification of the SOD extract. Precipitation obtained from centrifugation is an impurity substance from the extraction process (Nurdinia *et al.*, 2019).

Separation using centrifugation has a principle based on the size and weight of molecules, particles that settle at different speeds according to their weight, size, and shape. The sediment obtained is then washed using PBS, The use of PBS in this study is because it has the property of not interfering with the performance of enzymes in the interaction between protein bindings (Sri Wahjuni, 2015). The pellets obtained are then weighed to determine the required PBS weight in a ratio of 2:1. Pellets are sonicated at amplitude 50 for 15 minutes which functions to remove SOD in the cytoplasm produced in ribosomes. Pellets are sonicated at an amplitude of 50 for 15 minutes which aims to remove SOD in the cytoplasm with intervals every 1 minute at a temperature of 4oC. It is continued at the centrifugation stage again to obtain the extraction results which are then stored at refrigerator temperature.

SDS PAGE Results

The molecular weight of SOD using SDS PAGE is 30 kDa (Seatovic *et al.*, 2021) derived from thermophilic bacteria, according to research (Asadpour *et al.*, 2021) explains that SOD produced by bacteria *Eschericia Coli* has a molecular weight of 37 kDa. This is different from research (Tri Panji, *et al.* 2022) where the molecular weight of SOD with the appearance of two thin bands with a molecular weight of 19.20 kDa is suspected to be due to the relatively short denaturation. The molecular weight of the first and second SOD is the molecular weight of the SOD subunit resulting from the total SOD breakdown due to the use of SDS and mercaptoetanol in the electrophoresis process.

The SOD sub-unit with a molecular weight of 30 kDa contains up to 72% of the total metal, Fe 25%, while Mn and Cu are only 2% and 1%, respectively. Meanwhile, the SOD sub-unit with a molecular weight of 31.738 kDa contains Zn 55%, Mn 31%, Fe 14%, while Cu is only 4%. The Cu cofactor in SDS-PAGE appears up to 4%, while in SOD extracts from column chromatography it is not detected. This can be caused by the high concentration of SOD present in SDS PAGE, after being freed from phosphate buffer solvents and water (Gallagher, 2020).

Precipitation with Ammonium Sulfate

Protein levels and enzyme activity have the highest activity with 60% ammonium. In ammonium 80%, it shows a decrease in enzyme activity, this is due to the enzyme being saturated with ammonium sulfate or ammonium sulfate salts are bound to proteins, causing the value of enzyme activity to decrease (Rohishoh, 2012). Previous research by Bahri, *et al.* 2020 characterized enzymes using ammonium sulfate salts with a concentration of 60% and produced the highest enzyme activity. Research by Sriwahyuning, *et al.* 2019 used 60% ammonium sulfate salts to isolate enzymes and obtained different extract weights obtained from each sample due to environmental factors such as temperature, pH, and different types of samples. The principle of protein deposition with ammonium sulfate salts is *salting out*, the addition of salt with a high concentration can result in decreased protein solubility. The purity level of an enzyme is known from the specific activity of the enzyme, the higher the specific activity of an enzyme, the purer the enzyme will be (Brunelle & Green, 2014). Ammonium sulfate is used in the purification process because it is highly soluble, cheap and has the highest purification rate, and does not change the pH of the protein solution which can result in protein denaturation (Rosalina Suhito, 2019). The purification process using ammonium sulfate utilizes high salt content to precipitate proteins, protein solubility will decrease if it is in these conditions (Cutler, 2019).

The addition of ammonium sulfate to the saturated concentration is intended so that the collection of proteins when it has passed the saturation point. Some water molecules will be attracted by the salt ions of ammonium sulfate so that there is a decrease in water molecules to interact with the hydrophilic amino acids of the protein, so that proteins containing hydrophilic amino acids will settle (salting out). At low concentrations salt ions will protect the protein molecules, this event is called *salting in* (Scopes, 1987). The purification process is carried out in cold conditions to reduce

inactivation, where the temperature increases due to the dissolution process with the help of *magnetic stirrer* can cause denaturation. The deposition process is enhanced by storing the enzymes to which ammonium sulfate has been added overnight at a temperature of 4oC. During the storage process the protein molecules are aggregated but not all precipitate immediately, some of the protein aggregates float and collect on the surface forming a layer.

Protein Levels Measurement with the Lowry Method

Lowry's method is based on a biurete reaction with additional steps and reagents to improve detection sensitivity. In the biurete reaction, copper interacts with four peptide nitrogen atoms to form a copper complex. Lowry added phosphomolybdate/ phosphotungstat acid which is also known as *the Folin-Ciocalteu* reagent. This reagent interacts with copper ions and the side chains of tyrosine, tryptophan, and cysteine to produce a detectable blue-green color between 650 nm and 750 nm. The color formed is caused by an electronic transition that involves the transfer of valence electrons to other electrons. *The Folin-Ciocalteu* reagent for detecting reduced copper makes the Lowry test nearly 100 times more sensitive than the Biuret reaction. The concentration of ammonium sulfate affects the deposition of proteins. The higher the concentration of ammonium sulfate added, the more protein is deposited. The more protein that settles, the higher the protein content (Waterborg & Matthews, 1994). In the blue color Lowry method depending on the content of tryptophan and tyrosinase residues, the detection limit is around 0.01 mg/mL (Sudarmanto, 2008).

SOD Enzyme Activity with WST-1

This study found 4 bacterial isolates with an inhibition percentage of >50%, with the highest inhibition % of 86.57% belonging to BAL 06. SOD is an enzymatic antioxidant that facilitates the conversion of superoxide anions into hydrogen peroxide (H2O2) and oxygen (O2), thereby reducing ROS activity species (Najmuldeen, *et al*. 2019). Radicalism caused by these free radicals can be reduced with SOD antioxidants, which donate electrons so that they become stable. SOD functions through receptors that inhibit activation, as well as suppress the expression of AP-1 and MMP genes, thereby stimulating collagen production by increasing TGF-b genes and procollagen expression (Indrayati, *et al*. 2016). The activity test using the WST-1 method was able to inhibit superoxide anions up to 79.05%±2.62%. This is similar to the activity of 4 forms of SOD including copper-zinc (CuZnSOD), iron (FeSOD), manganese (MnSODA1 and MnSODA2) of bacteria *Klebsiella* which ranges from 51.67-82.82% (Najmuldeen, *et al*. 2019). This activity is expressed as units per milliliter of serum (U/mL). The principle of the WST-1 Xanthine Oxidase (XO) method can catalyze the reaction of WST-1 with O2- to dissolve in the water of the forman dye. SOD can catalyze the disproportion of superoxide anions, so that the reaction can occur inhibited by SOD, and SOD activity is negatively correlated with the amount of formant dye. Therefore, SOD activity can be determined by WST-1 analysis (Bannister) *et al.*, 1987). WST-1 has a mechanism to produce water-soluble formazan staining based on the reduction of superoxide anions.

CONCLUSION

SOD extract produced by seawater bacteria isolate of Pasir Putih beach, East Java, has a molecular weight of 30 kDa. SOD extract produced by seawater bacteria isolate from the Pasir Putih coast of East Java has a total protein content of 11.88 mg/mL. SOD extract produced by seawater bacteria isolate of Pasir Putih beach, East Java had activity with the highest inhibition % at BAL 06 of 86.07%.

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